Express Mail No.: EV335858897US

PATENT

UTXC509

APPLICATION FOR UNITED STATES LETTERS PATENT

for

DELIVERY AND ACTIVATION THROUGH LIPOSOME INCORPORATION OF DIAMINOCYCLOHEXANE PLATINUM(II) COMPLEXES

by

Roman Perez-Soler and Abdul R. Khokhar

EXPRESS MAIL MAILING LABEL

NUMBER EM219699721US

DATE OF DEPOSIT 12-6-96

I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington D.C. 20231.

Signature

H 150794(3ECQ01!DOC)

BACKGROUND OF THE INVENTION

The present invention relates to methods and compositions for the treatment of cancer.

One drug that has proven effective in the treatment of certain tumors is cisplatin (cis-dichlorodiamine-platinum(II)). However, cisplatin has certain disadvantages. For example, its use in some circumstances is limited by its toxicity to the patient, especially its nephrotoxicity. As another example, tumors sometimes develop resistance to cisplatin.

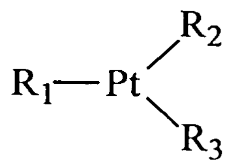
In an effort to overcome the disadvantages of cisplatin, researchers have synthesized and tested various other platinum complexes as potential antitumor agents. One such compound is dichloro(1,2-diaminocyclohexane) platinum(II) (referred to in the remainder of this patent as "DACH-Pt-Cl_2"). However, this compound has very low solubility in water, making it impractical for formulation and administration in aqueous solution. Further, although various platinum complexes have been formulated in liposomes in the past, a liposomal formulation of DACH-Pt-Cl_2 has not been developed because that complex is insoluble in most organic solvents. Although it has good solubility in dimethylformamide, that solvent has a very high boiling point, therefore making it impossible or impractical to prepare a liposomal formulation of DACH-Pt-Cl_2 using standard evaporation methods.

Other platinum-based antitumor drugs, such as cis-bis-neodecanoato-trans-R,R-1,2-diaminocyclohexane platinum (II) (NDDP) have been prepared and tested as antitumor agents. However, a need still exists for improved antitumor drug formulations that have good antitumor activity, low toxicity to non-cancerous cells in a patient, and desirable storage characteristics.

SUMMARY OF THE INVENTION

The present invention relates to a liposomal antitumor composition and to methods of using the composition to inhibit tumor growth in mammals. The invention also concerns methods of preparing the antitumor composition.

The present invention can take advantage of intraliposomal conversion of a platinum complex having the formula



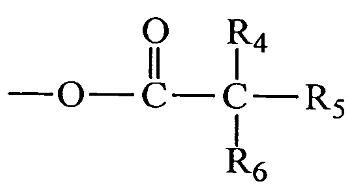
(I)

into a complex having the formula

R_1-Pt-X_2 (II)

where X is halogen. This makes it possible to prepare liposomal formulations of the complex (II), which had not been practical previously due to its low solubility in water and most organic solvents.

In the above complexes, R_1 is diaminocycloalkyl, preferably a 1,2-diaminocycloalkyl group having from about 3 to about 6 carbon atoms, most preferably 1,2-diaminocyclohexane. R_2 and R_3 each have the formula



where R_4, R_5, and R_6 are each independently hydrocarbon moieties having from 1 to about 10 carbon atoms, preferably alkyl having from 1 to about 6 carbon atoms, most preferably alkyl having from 1 to about 3 carbon atoms. R_2 and R_3 can be the same but do not have to be the same. Likewise, R_4, R_5, and R_6 can be the same but do not have to be the same. X is most preferably chlorine.

One aspect of the present invention is a liposomal antitumor composition that comprises the complex (II) entrapped in a liposome. In a particular embodiment of the invention, the liposome comprises an acidic phospholipid, for example dimyristoyl phosphatidyl glycerol. Without being bound by theory, it is believed that the presence of

the acidic phospholipid in the liposome enhances or accelerates the conversion of the complex (I) to the complex (II).

Another aspect of the present invention is a method of inhibiting tumor growth. The method comprises administering to a mammal a composition that comprises the complex (II) entrapped in a liposome, with the platinum complex being present in an amount effective to inhibit tumor growth.

Another aspect of the present invention is a method of preparing the antitumor composition. This method comprises the step of adjusting the pH of a composition that comprises the platinum complex (I) entrapped in a liposome, whereby the pH is made somewhat acidic, preferably between about 2 and about 6.5, resulting in the conversion of the complex (I) into the complex (II). The resulting composition can then be administered to a patient.

In one particular embodiment of this method, the complex (I) is converted to the complex (II) within the liposome. This allows a liposomal formulation of the complex (I) to be manufactured and stored, and then shortly before administration to a patient, the liposomal formulation of complex (I) can be converted to a liposomal formulation of complex (II) in situ by simply adding an acidic solution to the formulation. Optionally, after a predetermined time has passed since the addition of the acidic solution, the pH can be readjusted, preferably to at least about 7, in order to stop the conversion of complex (I) to (II).

A broader aspect of the invention concerns a method of delivering a biologically active chemical moiety internally to a mammal. The biologically active moiety can be, for example, an antitumor agent. The method comprises (a) providing an aqueous formulation of a prodrug of the biologically active moiety, the prodrug being entrapped in a liposome, and the prodrug further being capable of forming the biologically active moiety upon exposure to a solution having an acidic pH; (b) reducing the pH to an acidic level, thereby converting the prodrug to the biologically active compound; and (c) administering the aqueous formulation to a mammal. Administration of the liposomal formulation to the mammal can suitably be done after the conversion of the prodrug, but

it might also be done before conversion, such that the conversion would then occur in vivo, for example due to acidic components (e.g., acidic phospholipids) of the liposome.

The present invention has a number of advantages over prior art platinum antitumor formulations and methods, including better antitumor activity, greater potency, and reduced toxicity to non-cancerous cells of the patient. Further, the compositions of the present invention permit the formulation in liposomes and the delivery of platinum complexes that could not be so formulated in the past.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Chemical structure of NDDP, B10, and L10.

Figure 2. Effect of structure of Pt-complex, DMPG content, and aqueous solution on the intraliposomal stability and cytotoxicity of NDDP (✱), B10 (○), and L10 (■). Fig. 2A -- DMPC:DMPG=7:3, saline; Fig. 2B -- DMPC:DMPG=7:3, PBS; Fig. 2C -- DMPC:DMPG=3:7, saline; Fig. 2D -- DMPC:DMPG=3:7, PBS. IC_{50} values are in g/ml.

Figure 3. Effect of lipid composition on the intraliposomal stability of NDDP. 0.9% NaCl aqueous solution (starting pH=7.0) was used as reconstituting solution of liposomes and the final pH was checked at 6 h after liposome preparation.

Figure 4. ^{195}Pt NMR of liposomal NDDP suspension. Fig. 4A -- ^{195}Pt NMR of Sample 1 in chloroform prepared by extraction with $CHCl_3$ from liposomal NDDP suspension reconstituted in saline and kept for 6 h at room temperature. Fig. 4B -- ^{195}Pt NMR of Sample 2 in CH_3OH prepared by reconstitution of liposomal NDDP in saline for 6 h, lyophilization of water for 2 d, and redissolution of mixtures in CH_3OH. Fig. 4C -- ^{195}Pt NMR of Sample 3 in CH_3OH prepared by evaporation of t-butanol and redissolution of mixtures in CH_3OH.

Figure 5. Fig. 5A -- ^{1}H-^{1}H correlated spectroscopy of DACH-Pt-Cl_2 in DMF-d_7. Fig. 5B -- ^{13}C NMR of DACH-Pt-Cl_2 in DMF-d_7.

Figure 6. Sample preparation for NMR tracking experiment of liposomal NDDP. Fig. 6A -- with lipids in chloroform solutions. Fig. 6B -- with lipids in dry powder.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Cis-bis-neodecanoato-trans-R,R-1,2-diaminocyclohexane platinum (II) (NDDP) is a lipophilic platinum complex (Pt-complex) that can be formulated in a liposomal carrier.

Various details about the making and use of NDDP and other platinum complexes are disclosed in U.S. patents 5,041,581, 5,117,022, 5,186,940, 5,178,876, and 5,384,127. Those patents are incorporated here by reference.

Prior studies have suggested that NDDP is a pro-drug that exerts its biological activity through activation within the liposome bilayers. In order to understand the kinetics of the intraliposomal degradation/activation of different liposomal Pt-complexes, we studied the effects that the structure of the Pt-complex, pH, temperature, lipid composition, content of acidic phospholipids, liposome size, and presence of residual chloroform have on the stability, in vitro cytotoxicity, and in vivo antitumor activity of different liposomal Pt-complex preparations. The following factors were found to enhance the intraliposomal degradation/activation of Pt-complexes: 1) the size and spatial configuration of the Pt-complex, 2) an acidic pH, 3) a high temperature, 4) the presence and amount of acidic phospholipids, and 5) the presence of residual chloroform. Liposome size did not affect the intraliposomal stability of different Pt-complexes.

A good inverse relationship between the extent of drug degradation and in vitro cytotoxicity and between the extent of drug degradation and in vivo antitumor potency was observed, thus confirming that the biological activity of these complexes is exerted through the intraliposomal formation of certain active intermediate(s). The only active intermediate that could be identified was cis-bis-dichloro-trans-R,R-1,2-diaminocyclohexane platinum (II) whose structure was confirmed by ^{1}H, ^{13}C, and ^{195}Pt nuclear magnetic resonance (NMR) spectroscopy.

We have previously developed liposomal formulations of lipophilic Pt-complexes for in vivo administration [5,6] and have studied their chemical and biological properties. [1,4,7] The general structure of the preferred Pt-complexes used is [DACH-Pt-R_2], where DACH is trans-R,R-1,2-diaminocyclohexane and R is a lipophilic carboxylato group. The Pt-complex is thought to intercalate between the phospholipid molecules of the lipid bilayers of the liposomes. The most remarkable characteristic of these complexes is that they are not cross-resistant with cisplatin, both in vitro and in vivo. [1,5] The leading formulation, liposomal cis-bis-neodecanoato-DACH-platinum (II) (NDDP) uses large

liposomes composed of dimyristoylphosphatidyl choline (DMPC) and dimyristoylphosphatidyl glycerol (DMPG) at a 7:3 molar ratio and is now in clinical trials. Interestingly, liposomal-NDDP must undergo a chemical degradation/activation process into an active intermediate(s) within the liposomes shortly after liposome preparation in order to exert its antitumor activity. [7] We have previously reported that this chemical reaction depends on the content of DMPG in the lipid bilayer, and based on this finding, we hypothesized that a DMPG-Pt complex might be one of the active intermediates. We have also reported that the structure of the Pt-complex has an effect on the intraliposomal drug stability: the compounds with linear and short carboxylato leaving groups are more stable and less potent than the compounds with branched or longer linear leaving groups. [8] A full characterization of the active intermediate(s) as well as the different factors that influence the degradation/activation process is mandatory for the development of one of these agents as a pharmaceutical product.

For that purpose, we selected NDDP (highly branched structure) and two isomers, B10 (minimally branched structure) and L10 (linear structure) (Figure 1) and studied the relationship between their biological activity and their intraliposomal stability. We examined the effect of pH, temperature, lipid composition, liposome size, and presence of residual chloroform on the degradation of the Pt-complexes, and attempted to identify the active intermediate(s) by tracking experiments using ^{31}P and ^{195}Pt NMR spectra. Our results indicate that the degradation/activation of these Pt-complexes is greatly dependent on the pH of the suspension, and that DACH-dichloroplatinum (DACH-Pt-Cl_2) is the only intermediate that can be identified, thus suggesting that these Pt-complexes are prodrugs of DACH-Pt-Cl_2 when incorporated in liposomes suspended in saline.

MATERIALS AND METHODS

Preparation of liposomal Pt-complex.

NDDP, B10, and L10 were synthesized as previously described [1,2] and recrystallized in acetone. DMPC, DMPG, dioleyl phosphatidyl choline (DOPC), dioleyl phosphatidyl glycerol (DOPG), phosphatidic acid (PA), phosphatidyl ethanolamine (PE), and phosphatidyl serine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL).

Multilamellar vesicles containing Pt-complexes were prepared by the lyophilization method using the lipids in chloroform solutions or in dry powder.

Method 1: With chloroform solutions of lipids [1], lipids were mixed at the desired molar ratio, and the chloroform was removed in a rotary evaporator. To the dried lipid film, t-butanol solutions of Pt-complex were added and shaken at 40°C for 10 min. The solutions were then frozen in a dry-ice-acetone bath, and t-butanol was removed by lyophilization overnight to give lyophilized preliposomal powder.

Method 2: With lipids in dry powder, lipids were mixed and dissolved in t-butanol:water (10:1/v:v). To this solution, t-butanol solutions of Pt-complex were added, and the rest of the procedure was the same as described above.

Saline or PBS was added (1 ml/mg of Pt-complex) to reconstitute the lyophilized preliposomal powder, and the suspension was hand-shaken for 10 min to obtain large-size liposomes. Small-size liposomes were prepared by sonication of large-size liposomes for 1 min with an ultrasonic cell disrupter (Laboratory Supplies Co., New York, NY). The size distribution of the different liposomal preparations was determined with a Nicomp Submicron Particle Sizer Model 370 (Nicomp Particle Sizing Systems, Santa Barbara, CA).

Intraliposomal stability.

Stability of the different Pt-complexes incorporated in liposomes was determined as described previously [8] by comparing the HPLC profiles as a function of time. In brief, aliquoted samples of liposome suspension were diluted (7x) with methanol at 0, 2, 6, and 24 h after liposome preparation, and each sample was then monitored by HPLC using chromega-8 bond column (4.6mm x 25cm, 8 μm: ES Industries, NJ) and 10% water-methanol as eluant. The flow rate was 1 ml/min, and the complexes were detected by UV at 224 nm wavelength.

Biological activity.

In vitro. The in vitro cytotoxicity of liposomal Pt-complexes against A2780 human ovarian carcinoma cells was assessed by the MTT dye reduction assay. In brief, A2780 cells were seeded in 96-well plates, allowed to attach overnight, and then exposed

to various concentrations of drugs for 20 h. After washing the cells with PBS, fresh medium was added for 52 hours and the cell survival fractions determined by MTT assay.

In vivo. The in vivo antitumor activity of liposomal Pt-complexes was assessed against intraperitoneal L1210 mouse leukemia. Groups of 6-8 mice weighing 18-20 g were inoculated with 106 cells (0.2 ml, i.p.) on day 0, and treatment (25, 50, 100 and 150 mg/kg) was started on day 1 (0.15-0.5 ml, i.p.). The results were expressed as the median survival of treated animals divided by the median survival of control animals x 100 (% T/C).

Identification of active intermediates.

To characterize the active intermediate(s) in the reaction cascade of L-NDDP reconstituted in saline (0.9% NaCl), tracking experiments using ^{195}Pt NMR spectra in combination with ^{31}P NMR were performed. The procedures for the preparation of the samples is summarized in Fig. 6. Samples 1-3 were prepared using the lipids DMPC and DMPG purchased in chloroform solution, while samples 4-8 were prepared using lipids in dry powder. In samples 1-3, the chloroform was initially evaporated in a rotavapor. The lipid film was dissolved in t-butanol containing the NDDP in solution. An aliquot of this solution was kept at 40°C for 6 h and then lyophilized and extracted with methanol (sample 3). The remaining was lyophilized immediately, thus resulting in a preliposomal powder, which was reconstituted with saline to produce the liposome suspension. The liposome suspension was kept at room temperature for 6 hours. The Pt compounds and lipids were then extracted with chloroform (sample 1) or the sample was lyophilized to eliminate the water and the powder dissolved in methanol (sample 2) (see Fig. 6). Samples 4, 5, and 6 were prepared by complete evaporation of solvents after keeping the samples at 40°C for 6 h, and redissolving them in methanol. Samples 7 and 8 were prepared by lyophilization of water for 1-2 d and redissolution in methanol. All samples were prechecked by HPLC before tracking with NMR. Chemical shifts of the products are expressed in parts-per-million relative to Na_2PtCl_6 in ^{195}Pt and DMPC in ^{31}P NMR.

DACH-Pt-Cl_2 characterization.

Yellow precipitates from NMR samples were collected and redissolved in DMF-d_7 to characterize them with ^{1}H and ^{13}C NMR. ^{1}H NMR (DMF-d_7): 1.13-1.17 (m,

2H), 1.46-1.55 (m, 4H), 2.05-2.09 (broad, 2H) 2.55-2.59 (m, 2H), and 5.07 and 5.63 (broad s, 2 NH2) ppm. ^{13}C NMR (DMF-d7): 24.9 (C4, C5), 32.3 (C3, C6), and 64.1 (C1, C2) ppm. ^{195}Pt NMR ($CHCl_3$, CH_3OH) 1950 ppm, (DMF) 2250 ppm (strong single peak). Elemental analysis: Calc. C(18.99), H(3.69), N(7.38), Pt(51.50); Found C(18.58), H(3.72), N(7.40), Pt(51.30). All these data were confirmed by an authentic sample of DACH-Pt-Cl_2.

RESULTS

Preparation of liposomal Pt-complexes.

NDDP and its two isomers, B10 and L10 were formulated in liposomes composed of combinations of various lipids including DMPC, DMPG, DOPC, DOPG, PA, PE, and PS. The liposomes were formed by reconstituting preliposomal powders containing the Pt-complex and the lipids with unbuffered 0.9% NaCl aqueous solution (saline) or phosphate-buffered saline (PBS). The entrapment efficiency (%EE) of all liposomal formulations was >90% and was not significantly affected by the lipid composition, reconstitution solution, or NDDP isomer used. No crystals of free drug were observed in any of these preparations within 24 h as assessed by optic microscopy. The median size of the multilamellar vesicles was 1-2 μm in all preparations. The median size of the small-size liposomes prepared by ultrasonication of multilamellar vesicles was 50-100 nm.

Intraliposomal Stability of Pt-complexes.

1) Role of the spatial configuration of the Pt-complex and pH of the liposome suspension.

Figs. 2A-D show the stability of liposomal Pt-complex formulations using saline or PBS as the reconstitution solution and DMPC:DMPG ratios of 7:3 and 3:7. As observed previously [1], the branched configuration of the leaving group of the Pt-complex and the content of DMPG in the lipid bilayers correlated with a higher rate of degradation of the Pt-complex. As a result, the complex with a linear leaving group L10 was highly stable, while the highly branched NDDP was rather unstable, and the minimally branched B10 had an intermediate stability. The use of PBS as the reconstitution solution resulted in a significantly higher stability of the Pt-complexes as

compared with saline. For example, 6 h after liposome preparation, the percentages of intact NDDP in saline versus PBS were 43.7% vs 82.1%, whereas the percentages for B10 were 85.0% vs 95.9%, and the percentages for L10 93.1% vs 100%, respectively. The pH of the liposome suspension in saline decreased from 7.0 to 3.8-6.2 depending on the Pt-complex, whereas PBS held the pH of the solution to around 6.0-7.0 in all cases. These results indicate that: 1) an acidic pH enhances the intraliposomal degradation of the Pt-complexes, and 2) a good neutral buffer system can reduce or stop the intraliposomal degradation of the Pt-complexes. To confirm these results, we tested the intraliposomal stability of the Pt-complexes in strongly acidic (pH=3.0) or basic (pH=8.0) saline solutions prepared by adding 0.1 N HCl or NaOH aqueous solution to pH 7.0 saline. The pH 3.0 saline increased the degradation rate of all Pt-complexes, whereas the pH 8.0 saline did not induce any significant Pt-complex degradation even at 24 h after liposome preparation. All formulations using a relative higher amount of DMPG (DMPC:DMPG=3:7) displayed a higher rate of Pt-complex degradation in good correlation with the pH of the liposome suspension, because DMPG is an acidic phospholipid.

2) Role of temperature.

The intraliposomal stability of the Pt-complexes was checked at 40°C and compared with results obtained at room temperature. Pt-complex degradation was temperature-dependent, with the degradation rates being about 30-70% higher at 40°C than at 25°C, depending on the Pt-complex tested.

3) Role of lipid composition.

Liposomal formulations of NDDP using DMPC:PA, PS:DMPG, and DMPC:PE at a 7:3 molar ratio, and DOPC:DOPG at 1:0, 7:3, 3:7, and 0:1 molar ratios were prepared and tested using saline as the reconstitution solution (Figure 3). The acidity of phospholipids (PA>PG>PS) and the relative DOPG content (DOPC:DOPG 0:1>3:7>7:3>1:0) enhanced the intraliposomal degradation of NDDP and a good correlation between Pt-complex degradation and acidic pH was again observed.

The same conclusion was drawn in studies with NDDP in liposomes with the same DMPC:DMPG molar ratio (7:3) but different NDDP:total lipid ratios (1:5, 1:10,

1:15, and 1:30) and, therefore, different DMPG contents. Using a 1:5 or 1:10 ratio, 85% of initial NDDP was present at 24 hours; in contrast, using a 1:15 or 1:30 ratio resulted in an enhanced NDDP degradation, with only 25% of the original NDDP remaining at 24 h, thus suggesting a correlation between extent of degradation and absolute amount of DMPG within the lipid bilayers.

4) Role of liposome size.

Liposome size did not affect the intraliposomal stability of NDDP: ultrasonication of the original suspension of multilamellar vesicles did not significantly change the stability of NDDP regardless of the lipid composition used.

Correlation between in vitro cytotoxicity and intraliposomal stability.

We studied the in vitro cytotoxicity of different liposomal Pt-complex preparations against A2780 cells with the MTT assay and correlated the results with the intraliposomal stability of the Pt-complex. Results are shown in Figs. 2A-D. The IC_{50} values correlated fairly well with drug stability: the more stable the Pt-complex, the less toxic or higher the IC_{50}. When about 20%, 50%, and 90% of the original Pt-complex remained at 6 h, the IC_{50} was approximately 3-5, 7-10, and 20-50 g/ml, respectively. These results indicate that the intraliposomal degradation of the Pt-complex is required to exert its cytotoxic effect and is, therefore, an intraliposomal activation step.

Identification of active intermediate(s) of NDDP.

No new peaks corresponding to the degradation products are observed by the HPLC method developed for NDDP, either because they elute with the phospholipids or they do not have UV absorbence. Attempts to separate any new peaks from the lipid peaks have been so far unsuccessful.

To keep track of the reaction cascade of liposomal NDDP, we tried to apply the NMR tracking technique used by other researchers [2, 3, 9-11] to characterize the degraded/activated products of NDDP.

1) Formulations prepared using lipids in chloroform solution (samples 1, 2, and 3 of Fig. 6A).

By ^{195}Pt NMR of sample 1 (saline, chloroform extraction, Figure 4A) and sample 2 (saline, lyophilization, Figure 4B), the NDDP peak was detected at 1750 ppm and a

new peak corresponding to DACH-Pt-Cl_2 was detected at 1950 ppm. Prolonging the reaction time, lowering the pH, increasing the temperature, and increasing the amount of DMPG in the liposomes enhanced the degradation/activation of NDDP, increasing the intensity of the peak at $^{195}0$ ppm. However, by ^{31}P NMR, no new peaks were observed except those corresponding to DMPC (2 ppm) and DMPG (3 ppm), indicating that the new product shown by ^{195}Pt NMR is not a DMPG-incorporating Pt-complex.

These spectra results were similar to those from sample 3 (Fig. 6A, t-butanol, lyophilization, Figure 4C), in which liposomal NDDP was not exposed to saline, thus indicating that the presence of $CHCl_3$ can also act as a donor of chloride to form DACH-Pt-Cl_2. After storing the yellowish NMR samples at 4°C overnight, yellow crystals slowly precipitated. The precipitates were filtered, dried, and the structure of the compound was proved to be DACH-Pt-Cl_2 by ^1H, COSY (Figure 5A), ^{13}C (Figure 5B), and ^{195}Pt NMR. A yellow precipitate was also observed 2-3 weeks after leaving the original liposomal NDDP suspension at room temperature. NDDP in liposomes composed of only DMPC did not give any new peaks by either ^{195}Pt or ^{31}P NMR, which correlates with its completely preserved stability in the absence of DMPG.

2) Formulations prepared using lipids in dry powder (samples 4, 5, 6, 7, and 8).

We performed the same tracking experiment with formulations prepared using lipids in dry powder instead of chloroform solutions to eliminate the influence of the presence of residual $CHCl_3$ on the degradation of NDDP (Fig. 6). In samples 4 and 5 (solvent t-Butanol + water and methanol, respectively) containing lipids and NDDP, no reactions occurred, whereas sample 6 (chloroform solution of lipids and NDDP) showed the presence of DACH-Pt-Cl_2 by ^{195}Pt NMR. The results with these samples, which were incubated at 40°C for 6 h, confirm that the presence of chloroform can induce the degradation of NDDP into DACH-Pt-Cl_2. Sample 7 was prepared by reconstitution of preliposomal NDDP powder in saline of pH 6.5-7.0 for 6 h at room temperature. No significant degradation (<5%) was observed, whereas when reconstituted in acidic saline of pH 3.0-4.0 (sample 8), a 60-95% degradation of NDDP occurred in 10 min yielding DACH-Pt-Cl_2 as determined by ^{195}Pt NMR and HPLC (Table 1).

Table 1

Effect of various pH values of saline on intraliposomal stability of NDDP prepared with lipids as dry powder.

pH of saline	NDDP stability (%)			
	Time after reconstitution			
	10 min	1 h	2 h	6 h
3.0	43	--	15	7
5.0	80	72	67	38
7.0	100	--	95	92

Relationship between Pt-complex stability and in vivo antitumor activity.

1. Preparations containing residual chloroform

These studies were done with liposomal Pt-complex suspensions prepared using lipids dissolved in chloroform. In in vivo antitumor activity studies against L1210 leukemia, an inverse relationship between drug stability and antitumor potency was observed (Table 2). The DMPC:DMPG ratio was 7:3 for all preparations. The starting pH value of the saline and PBS was 7.0. The tumors were inoculated i.p. on day 0, followed by drug injection i.p. on day 1.

Table 2

In vivo antitumor activity of liposomal Pt complexes against L 1210 leukemia.

Pt complex	Dose of Pt complex (mg/kg)	%T/C Reconstitution solution Saline	PBS
NDDP	25	157	114
	50	211	171
	100	Toxic	200
	150	Toxic	150
B10	25	114	114
	50	128	114
	100	228	142
	150	163	163
L10	25	100	114
	50	157	100
	100	178	100
	150	123	100
Cisplatin	10	142	

The optimal doses of liposomal NDDP were 50 mg/kg in saline and 100 mg/kg in PBS (%T/C = 211 and 200, respectively). For liposomal B10 and L10, the optimal dose was 100 mg in saline (%T/C = 228 and 178), but no significant antitumor activity was observed when both drugs were reconstituted in PBS. In conclusion, the most potent liposomal Pt-complex preparations are those with the lowest stability of the Pt-complex. However, all formulations had similar antitumor activity when administered at the optimal dose.

2. Preparations not containing residual chloroform

These studies were done with liposomal Pt-complex suspensions prepared with dry lipids. The relationship between NDDP stability and antitumor activity was again

studied using the in vivo L1210 leukemia model. Table 3 shows the results with formulations reconstituted with saline solutions of different pH and administered at different time points after reconstitution. (Values are the means of two separate experiments.)

Table 3

Antitumor activity of liposomal NDDP against L 1210 leukemia.

Drug	pH of saline	Dose of Pt complex (mg/kg)	--------%T/C-------- Time of drug administration		
			10 min	2 h	6h
NDDP	3.0	12.5			157
		25	150	186	240
		50			Toxic
NDDP	5.0	12.5			186
		25			200
		50	133	216	270
		100			Toxic
NDDP	7.0	25			186
		50			200
		100	163	216	257
Cisplatin		10			150

The optimal doses of liposomal NDDP reconstituted with salines of pH 3.0, 5.0, and 7.0 were 25, 50, and 100 mg/kg, respectively. Therefore, the lower the pH, the higher the potency of the preparation in good correlation with the increased intraliposomal drug degradation/activation. At the optimal doses, the %T/C obtained were 214, 271, and 271, respectively. Delaying the time of drug administration increased the antitumor activity of the formulations, in good correlation with the increased drug activation with time. However, antitumor activity did not correlate perfectly with the calculated amount of activated Pt-species formed intraliposomally during the activation process. For example, 100 mg/kg at pH 7.0 gave a similar %T/C value as 50 mg/kg at pH 5.0, although only 10% of NDDP at pH 7.0 (10 mg/kg) and about 70% at pH 5.0 (35 mg/kg) are transformed into the active Pt-species under those conditions. Further in vivo activation must, therefore, occur to explain these discrepancies.

DISCUSSION

Our results indicate that NDDP and its isomers are prodrugs of DACH-Pt-Cl_2 when entrapped in liposomes containing acidic phospholipids and in the presence of sodium chloride or residual chloroform as donors of chloride. The rate of transformation of NDDP into DACH-Pt-Cl_2 is directly related to the pH of the liposome suspension. The studies performed suggest that DMPG and other acidic phospholipids enhance the reaction by providing an acidic milieu within the liposome membranes. No evidence could be generated to support a direct reaction between NDDP and DMPG to form a DACH-Pt-DMPG complex as one of the active intermediates of NDDP, as we had previously hypothesized, nor the formation of DACH-Pt aquated species.

DACH-Pt-Cl_2 is the leading compound of the DACH family of Pt-complexes. However, it was never developed because of a lack of solubility in water. We initially considered this compound for liposome entrapment but determined it to be an inappropriate drug for liposome formulation because it is insoluble in most organic solvents. DACH-Pt-Cl_2 has only a good solubility in dimethylformamide (DMF), which has a very high boiling point and, therefore, can not be used to prepare liposomes using the standard evaporation methods and it is not soluble in any of the organic solvents used for the lyophilization methods. Our studies indicate that DACH-Pt-Cl_2 can be generated

within the liposome membranes under the conditions described and that the drug remains liposome-bound without leaking out and crystallizing for at least 24 h. In contrast, DACH-Pt-Cl_2 precipitates quickly when formed from NDDP by the addition of HCl. These results constitute the first example of a liposome formulation in which the compound is synthesized in situ from an entrapped precursor and the liposomes prevent its spontaneous precipitation. The results are encouraging because they may suggest an avenue for the development of a much needed delivery system for this very interesting compound.

A potential strategy is to use a two-step reconstitution procedure by which an acidic saline solution is used first to induce the fast transformation of >80% of NDDP into DACH-Pt-Cl_2, followed after a predetermined period of time by the addition of a buffer solution to bring the pH to >7.0 and to stop the reaction.

Liposomes in accordance with the present invention can be prepared from various amphipathic substances including natural or synthetic phospholipids. Numerous suitable phospholipids are well known in the art. The liposomes of the present invention can be multilamellar, unilamellar, or have an undefined lamellar construction. A pharmaceutical composition comprising such liposomes can include a pharmaceutically acceptable carrier or diluent, as well as other pharmaceutically acceptable adjuvants.

Liposome compositions of the present invention can be used to inhibit the growth of tumor cells in mammals, particularly in humans. The compositions of the present invention should be useful for treatment of various human malignancies, in particular any platinum-sensitive cancer, including ovarian, testicular, lung, head and neck, esophageal, and bladder tumors, sarcomas, lymphomas, and mesotheliomas. Methods of using the compositions of the present invention involve administering to a mammal an amount of the compositions effective to inhibit tumor growth. The administering step can suitably be parenteral and by intravenous, intraarterial, intramuscular, intralymphatic, intraperitoneal, subcutaneous, intrapleural, or intrathecal injection, or by topical application or oral dosage. Such administration is preferably repeated on a timed schedule until tumor regression or disappearance has been achieved, and may be used in

conjunction with other forms of tumor therapy such as surgery or chemotherapy with different agents. The dose administered of a composition in accordance with the present invention is preferably between approximately 100 and 750 mg/kg of body weight of the mammalian subject to which it is administered.

The preceding description of specific embodiments of the present invention is not intended to be a complete list of every possible embodiment of the invention. Persons skilled in this field will recognize that modifications can be made to the specific embodiments described here that would be within the scope of the present invention.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

1. Han, I.; ling, Y.-H.; Al-Baker, S.; Khokhar, A.R.; Perez-Soler, R. Cellular pharmacology of liposomal cis-bis-neodecanoato- trans-R,R-1,2-diaminocyclohexane- platinum(II) in A2780/S and A2780/PDD cells. Cancer Res. 1993, 53, 4913-4919.
2. Hollis, L. S.; Miller, A. V.; Amundsen, A. R.; Schurig, J.E.; Stern, E.W. cis-Diamineplatinum(II) complexes containing phosphono carboxylate ligands as antitumor agents. J. Med. Chem. 1990, 33, 105-111.
3. Ismail, I. M.; Sadler, P. J. ^{195}Pt- and ^{15}N-NMR studies of antitumor complexes. In: Platinum, gold, and other metal chemotherapeutic agents; Lippard, S. J. (eds.) American Chemical Society. 1983; pp 171-189.
4. Khokhar, A.R.; Al-Baker, S.; Brown, T.; Perez-Soler, R. Chemical and biological studies on a series of lipid-soluble (trans-(R,R)- and -(S,S)-1,2-diaminocyclohexane) platinum(II). J. Med. Chem. 1991, 34, 325-329.
5. Perez-Soler, R.; Yang, L.Y.; Drewinko, B.; Lauterzstain, J.; Khokhar, A.R. Increased cytotoxicity and reversal of resistance of cis-diamminedichloro platinum(II) with entrapment of cis-bis-neodecanoato- trans-R,R-1,2-diaminocyclohexaneplatinum(II) in multilamellar lipid vesicles. Cancer Res. 1988, 48, 4509-4512.
6. Perez-Soler, R.; Khokhar, A. R.; Lautersztain, J.; Al-Baker, S.; Francis, K.; Macias-Kiger, D.; Lopez-Berestein, B. Clinical development of liposomal platinum. J. Liposome Res. 1990, 1, 437-449.
7. Perez-Soler, R.; Khokhar, A. R. Lipophilic cisplatin analogues entrapped in liposomes: role of intraliposomal drug activation in biological activity. Cancer Res. 1992, 52, 6341-6347.

8. Perez-Soler, R.; Han, I.; Al-Baker, S.; Khokhar, A. R. Lipophilic platinum complexes entrapped in liposomes: improved stability and preserved antitumor activity with complexes containing linear alkyl carboxylato leaving groups. Cancer Chemother. Pharmacol. 1994, 33, 378-384.

9. Qu, Y.; Farrell, N. J. Effect of diamine linker on the chemistry of bis(platinum) complexes. A comparison of the aqueous solution behavior of 1,4-butanediamine and 2,5-dimethyl-2,5-hexanediamine complexes. J. Inorg. Biochem. 1990, 40, 255-264.

10. Qu, Y.; Farrell, N. J. Interaction of bis(platinum) complexes with the mononucleotide 5'-guanosine monophosphate. Effect of diamine linker and the nature of the bis(platinum) complex on product formation. J. Am. Chem. Soc. 1991, 113, 4851-4857.

11. Slavin, L. L.; Bose, R. N. Phosphonato complexes of platinum(II): kinetics of formation and phosphorus-31 NMR characterization studies. J. Inorg. Biochem. 1990, 40, 339-347.